Trends in Parasitology

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Review

Sex Sorting for Pest Control: It's Raining Men!

Célia Lutrat,[1,2,3] David Giesbrecht,[4] Eric Marois,[5] Steve Whyard,[4] Thierry Baldet,[1,2] and Jérémy Bouyer[1,6,*]

In the pursuit of better pest- and vector-control strategies, attention returns to an old proven technology, the sterile insect technique (SIT) and related insect population-suppression methods. A major obstacle for any of these approaches that involves the release of sterile males is the separation of males from females during the mass rearing stage, in order to improve the cost-efficiency of these methods and to prevent the release of biting and disease-vectoring females. This review describes recent sex-sorting developments in dipteran flies with an emphasis on assessing the suitability of these methods for large-scale rearing of male vectors for mass release.

Highlights

Sexing Diptera represents a major obstacle to operationalizing vector-control methods based on the mass release of males, such as the sterile insect technique or the incompatible insect technique.

The recent progress made in gene editing, as well as the growing understanding of sex-determination pathways in Diptera, offer new perspectives to develop sexing systems in target species that allow female elimination during mass rearing.

The developmental stage at which the sexes are separated, or females are eliminated, is one of the key parameters for assessing the cost efficiency of a sexing technology.

Challenges remain in the development of sex-separating systems that are used early in mass rearing, without major genetic defects or lack of competitiveness in produced males, as well as good social and regulatory acceptability of the methods used, considering their operational deployment in the field.

Sexing Is an Obstacle in Genetic Pest-control Programs

Disease-vectoring Diptera are responsible for millions of parasitic and viral infections in humans and livestock annually [1]. As a more environmentally friendly alternative to broad-spectrum chemical insecticides, many researchers have been inspired by Knipling's proposal in 1955 [2] to develop methods of releasing sterile males to reduce pest insect populations. This so-called **sterile insect technique** (**SIT**) (see Glossary) has proven effective for a variety of insects, but its implementation is slowed down by the necessity of removing females before release in the case of mosquitoes. In addition to minimizing the health and economic risks posed by released females, models and trials have also shown that releasing only males was much more cost-efficient than releasing both sexes [2,3]. These cost savings may arise from either reduced cost in mass-rearing the insects and/or in field performance, where released males will not be distracted by coreleased females.

Other genetic control methods, including **release of insects carrying a dominant lethal** (**RIDL**) and the *Wolbachia*-based **incompatible insect technique** (**IIT**), also require consistent sexing methods. Models show that the release of only a small proportion of *Wolbachia*-infected females could lead to population replacement instead of elimination[i]. In mosquitoes, whose females cause nuisance and transmit pathogens, very little female contamination can be tolerated in any genetic control strategy.

In these applications, sex sorting, or 'sexing', refers to the separation of males from females, and more specifically the removal of females. Sexing can rely on mechanical separation of the sexes based on natural or engineered sexually dimorphic differences, or sexing can use more complex technologies to modify gene expression and conditionally masculinize or kill females during development. Overall, sex-separation strategies need to meet several criteria, summarized as 'the 7 Ses' by Papathanos and colleagues [4]: small, simple, switchable, stable, stringent, sexy, and sellable.

Sexing developments have been reviewed numerous times [5–8], focusing mostly on particular species or genera, as well as on the engineering methods employed. Here, we aim to review all sexing methods developed recently in Diptera. We have chosen not to focus solely on vector species since there has been a number of interesting technical developments in other dipteran insects that could complement technologies for mosquitoes and other disease vectors. With an

[1]CIRAD, UMR ASTRE, F-34398, Montpellier, France
[2]ASTRE, CIRAD, INRA, Univ Montpellier, Montpellier, France
[3]Université de Montpellier, Montpellier, France
[4]Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada
[5]CNRS UPR9022, INSERM U963, Institut de Biologie Moléculaire et Cellulaire, Université de Strasbourg, Strasbourg, France
[6]Insect Pest Control, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency (IAEA), Vienna, Austria

*Correspondence:
jeremy.bouyer@cirad.fr (J. Bouyer).

emphasis on an operational perspective of sexing methods, we explored advantages and disadvantages of each innovation in a mass-rearing context compared to what is currently being done. Our survey starts in 2003, as the most recent comprehensive review of all available insect sexing strains was published in 2002 [9].

Current Sex-Sorting Status in Diptera Mass Releases

Most current-day genetic control programs target pest flies for which sex-sorting is not always an option. For instance, the Australian and Thai *Bactrocera* mass-rearing facilities produce tens of millions of fruit flies per week without removing females [10,11]. Similarly, the screwworm *Cochliomyia hominivorax* has been targeted for years by weekly releases of 15 million sterile flies at the Panama–Colombia border (reviewed in [12]), without mass sex-separation strategy. With this scale of production, a sexing method at the pupal stage is being considered [13], with predicted savings of over US$ 1 million per year. To the authors' knowledge, the Mediterranean fruit fly *Ceratitis capitata* and the Mexican fruit fly *Anastrepha ludens* are amongst the only flies of agricultural importance for which sexing strains are used in mass-rearing plants around the world, improving greatly their release efficiency[ii] [14].

Where sex-sorting is mandatory, time-consuming approaches are often the only available option. Culicinae mosquitoes (including the genera *Aedes* and *Culex*) exhibit size **sexual dimorphism** as pupae. Consequently, *Aedes* mosquitoes have been, and are still, mechanically separated based on pupal size [15]. In Italy, a SIT trial to control *Ae. albopictus* was initiated in 2005 [16]. Over a 4-year period, 2 million males were sex-sorted with metal sieving plates before release. This method recovered only 26–29% of the males, and female contamination was still about 1.2%. In China, Fay-Morlan glass sorters, also based on size, enabled the release of more than 197 million *Ae. albopictus* males in 2016 and 2017 for a combined IIT/SIT trial in two river island settings, with greater male recovery, and female contamination about 0.3% [17]. Similar sexual dimorphism-based methods are being deployed for Dengue control on La Reunion Island in a trial against *Ae. albopictus*[iii] and in French Polynesia against *Ae. aegypti* and *Ae. polynesiensis*[iv,v]. In *Anopheles* mosquitoes, the current sex separation method is based on manual pupal identification, which allows sex-sorting of only 500 pupae per hour (reviewed in [15]). The working time is therefore very high, and the number of mosquitoes necessary for a program is difficult to reach. In tsetse flies, both sexes feed exclusively on blood and can act as vectors of trypanosomes. Release of sterile trypanocide-treated males demands low-throughput manual separation of chilled adults or the use of pupal **protogyny** [18,19]. During the elimination program of *Glossina palpalis gambiensis* in Senegal, 5 million males were produced using a protogyny-based sorting method [19].

Sex-sorting at the pupal or adult stage requires rearing and feeding both male and female larvae, the latter being discarded to retain only males. Moreover, increasing densities of larvae would reduce fitness and slow down development [20]. Therefore, removal of females early in development is advantageous to avoid competition between males and females [21]. When rearing millions of flies, early sex-separation translates into major savings in time, labor, and money and also decreases the risks of female mosquitoes feeding on workers in the mass-rearing facility.

For these reasons, we will review sex-sorting methods by distinguishing two categories: removal of females during the first larval stages and later in development.

Sex Separation Methods in Early Larval Stages

Disruption of the sex-determination pathway has been explored in many pest insects, with the goal of identifying genes that are essential for female development. The *Drosophila melanogaster*

Glossary

Genetically modified organism (GMO): European legal definition: 'an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination'[vi]. As sterile insects are not considered as organisms (see 'Organism'), they are not considered GMOs. Even with residual fertility, irradiated sterile male insects are exempted from GMO regulations when they are obtained by mutagenesis techniques that have conventionally been used in a number of applications, have a long safety record, and do not involve the use of recombinant nucleic acid molecules. However, on the international stage, in the Cartagena Protocol on Biosafety to the Convention on Biological Diversity, the definition of 'living modified organism' includes sterile organisms.
Incompatible insect technique (IIT): the IIT relies on the property of the bacterium *Wolbachia* to confer cytoplasmic incompatibility on its host. When crossed with infected females, *Wolbachia*-infected male mosquitoes sire infected offspring, but when mating with uninfected females they cannot sire any progeny. IIT is the repeated release of infected males in noninfected populations, resulting in a reduction of the target population.
Organism: European legal definition: 'any biological entity capable of replication or of transferring genetic material'[vi]. According to this definition, sterile insects are not organisms. Canadian Environmental Protection Act, 1999: an organism refers to a living organism, that is, 'a substance that is an animate product of biotechnology'.
Protandry/protogyny: in insects, protandry is the fact that males emerge before females while protogyny is the opposite.
Release of insects carrying a dominant lethal (RIDL): although considered as SIT by some authors, it does not fully fit the definition since released males are still fertile but transmit a lethality gene.
Sexual dimorphism: differences other than the sexual organs between sexes of the same species.
Sterile insect technique (SIT): according to the International Standards for Phytosanitary Measures No. 5 Glossary of phytosanitary terms, the

system has been used as a template for inquiry of other sex-determination cascades. However, even within closely related species, exploration of the primary signal and downstream factors has revealed major differences. Box 1 reviews our current understanding of primary sex-determination signals and downstream factors in Diptera. These genes can be manipulated to produce male-biased populations early in the insect's development (Table 1). Although only a few of them have actually been applied to mass rearing conditions, we classified these innovations according to their outcome, as an indicator of their potential efficiency.

sterile insect technique is a 'Method of pest control using area-wide inundative releases of sterile insects to reduce reproduction in a field population of the same species'.

Achieving Sex-Ratio Distortions

Theoretically, the most cost-efficient way of producing a male-only population is to produce male-only eggs. This was achieved in *C. capitata*, with a transgenic strain expressing a double-stranded RNA targeting *transformer* (see Figure S1 in the supplemental information online for an overview of the genetic methods discussed) under a heat-shock promoter. Following a transient heat shock, it produced offspring composed of 95% males and 5% intersex individuals [22]. In this study, most genetic females (XX) developed as phenotypic fertile males. Another promising sex-ratio distortion system is under development in *Anopheles* mosquitoes [23–25]. However, its expression has not yet been rendered conditional, a prerequisite for its use in making stable sexing strains.

Automated Sex-Separation in Early Larval Stages

The second most cost-efficient way to produce a male-only population would be to separate sexes early in development, either by conditionally killing the females or by removing them using sex-specific differential expression of fluorescent marker transgenes. Catteruccia *et al.* [26] established that the sperm-specific β2-tubulin promoter driving the expression of enhanced green fluorescent protein (EGFP) allows the identification of male *An. gambiae* mosquitoes as 4th instar larvae, as well as the sorting of male vs. female larvae using a complex parametric analyser and sorter (COPAS) flow cytometry machine. While this strategy enabled automated sorting in late larval stages, Magnusson *et al.* [27] developed another sex-sorting marker acting as early as the 1st instar stage. Males of this strain show strong EGFP expression from a reporter gene harboring a female-specific *dsx* intron. This strain allowed Marois and colleagues [28] to optimize

Box 1. The Sex Determination Pathways in Diptera

Sexual dimorphism in insects is controlled by diverse mechanisms to determine sex and differentiate sexual morphologies. Figure I illustrates what is known about dipteran sex-determination pathways.

Sex-determination mechanisms in Diptera range from what is familiar to many: dosage compensation of X and Y chromosomes in *Drosophila melanogaster* [83] to the homomorphic chromosomes of *Aedes aegypti*, in which transcription of the autosomal gene *nix* appears to be the maleness (M) factor initiating the determination of male mosquitoes [84]. Rapid evolution of these factors has produced a high degree of variation in the function of the genes involved, with marked differences described between closely related species [85].

What is common to all Diptera studied to date is the central regulator *doublesex* (*dsx*), a gene with female-specific exons and transcripts that have been targeted to induce female sterility or lethality [39,86,87]. *Dsx* is a transcription factor in the *doublesex/mab-3 related* gene family. Recently reviewed by Kopp [88], this gene family appears to be conserved in arthropods, but the diversity of roles that *dsx* plays in other lineages [89] suggests that it has been frequently coopted to new roles. Briefly, male or female *dsx* is expressed in tissues that require sexual identity. In *Drosophila melanogaster*, *dsx* is regulated by alternative splicing of *transformer* (*tra*), another conserved gene that has been successfully targeted for female lethality, which, in turn, is regulated by *sex-lethal* (*sxl*) [83]. In the muscids studied to date, only *tra* is known to be an upstream regulator of *dsx* [85]. In the mosquito species studied so far, only the male determining factors *nix* (*Aedes aegypti*) [90], *yob (An. gambiae)* [40] and *guy1* (*An. stephensi*) [91] are known or presumed regulators of *dsx*, but there may be other upstream factors regulating *dsx*. A role for *tra-2* in mosquito sperm development has been shown, resulting in reduction of female offspring in the second generation after RNAi knockdown [38]. Intriguingly, putative *tra/tra2* orthologues appear to be highly conserved in mosquitoes [38], but *tra/tra2* has not been implicated as a regulator of *dsx* in any mosquito, although *tra-2* is involved in ovarian development in *Ae. albopictus* [92]. The M-factor in tephritid flies has recently been described and shown to regulate *tra*'s auto-regulatory positive-feedback loop [93]. Similarly, in two phlebotomine sandflies, *tra* has been recently identified and shown to also be self-regulating [94]. There are likely to be many new opportunities to distort sex ratios by discovering players in the diverse pathways that have evolved in Diptera.

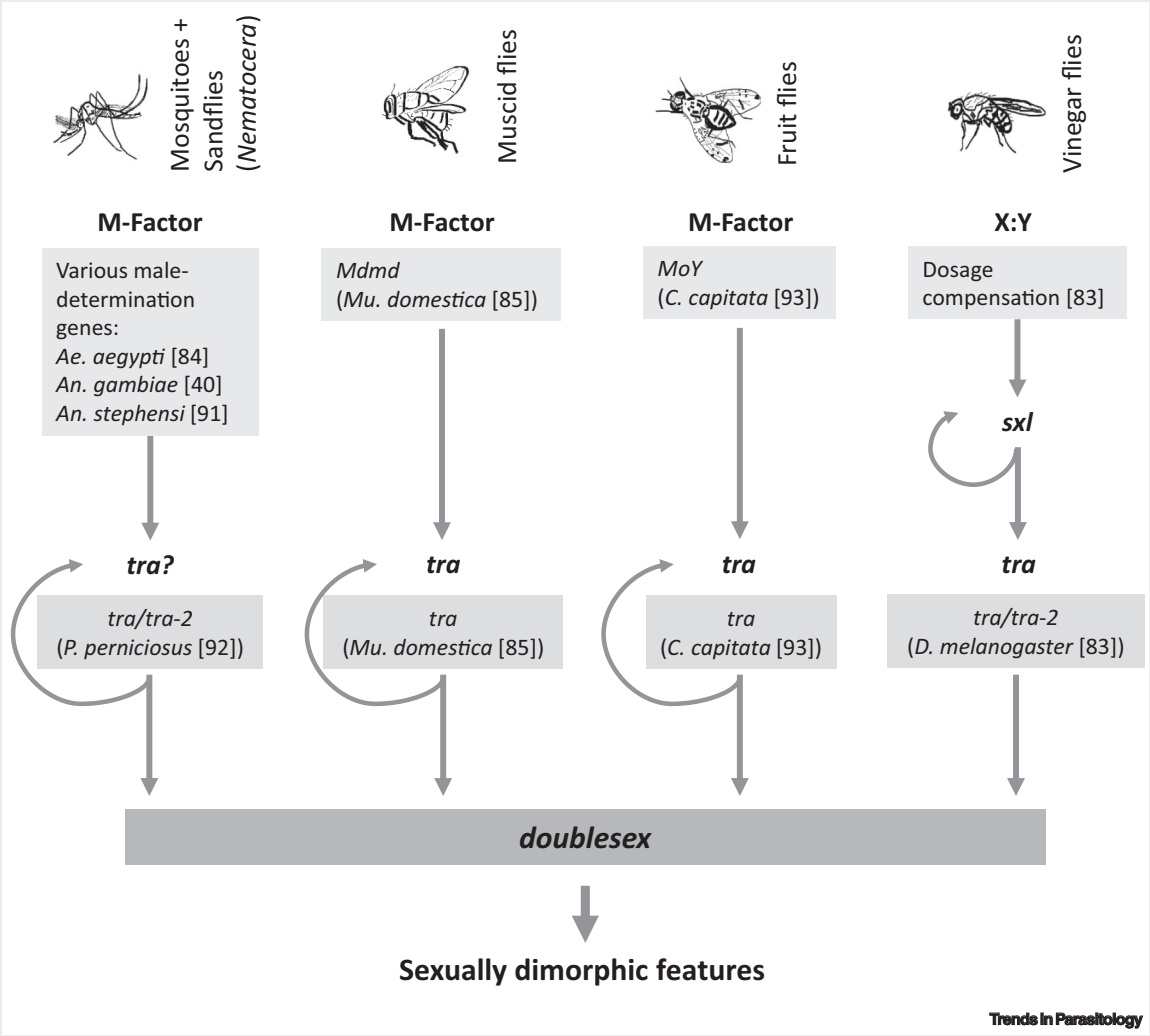

Figure I. Sex Determination Pathways in Diptera.

sex-sorting at high flow rate by COPAS. This study showed that pure male populations of 20 000 neonate larvae could be generated in 30 min, a rate that remains below the production scale required for mass rearing. Moreover, a GFP-expressing transgene inserted on the *An. gambiae* Y chromosome has been isolated, and additional strains were subsequently derived that expressed red fluorescence [29]. In this work, Bernardini and colleagues observed that the GFP strain enabled COPAS-based sorting of virtually 100% pure male mosquitoes. In locations where the release of transgenic **organisms** must be avoided, two sexing strains (one carrying a marked X chromosome, the other a marked Y) could be used in crossing-sorting schemes that generate a pure population of nontransgenic males (Figure 1). Of note, Bernardini *et al*. [30] were able to introgress one of their fluorescence-expressing Y chromosomes from *An. gambiae* to *An. arabiensis*, opening the possibility of automated sorting to other members of the *An. gambiae* species complex. This *An. arabiensis* strain is presently under testing in mass-rearing conditions at the FAO-IAEA Insect Pest Control Laboratory.

Table 1. Early Acting Methods for Sexing in Diptera since 2003

Outcome	Technique[a]	Sorting mechanism	Species	Refs
Sex ratio distortion	Interfering RNA	Silencing of *transformer (tra)* and *transformer-2 (tra-2)*	*Ceratitis capitata*	[22]
Visual separation	Transposase-mediated plasmid integration	RFP or GFP marker + COPAS	*Anopheles gambiae, Anopheles arabiensis*	[28–30]
		tTA system using *tra* intron and fluorescent marker	*Lucilia cuprina*	[31]
Female lethality	Transposase-mediated plasmid integration (*piggyBac*)	tTA driving expression of proapoptotic transgene	*Anastrepha suspensa, C. capitata, L. cuprina*	[32–34]
	Interfering RNA	Silencing of *tra* and/or *tra-2*	*Bactrocera dorsalis, Aedes aegypti*	[37,38]
		Silencing of *dsx*	*Ae. aegypti*	[39]
	mRNA injection	Overexpression of *Yob* by injecting mRNA	*An. gambiae, Anopheles arabiensis*	[40]
Intersex females	Transposase-mediated plasmid integration (*piggyBac*)	Plasmid injection causes overexpression of *Yob* under vasa2 promoter	*An. gambiae*	[41]
	CRISPR-Cas9 knockdown	Double knockdown of *tra-2*	*A. suspensa*	[42]

[a]Techniques are detailed in Figure S1 in the supplemental information online.

Sexing is equally important for livestock pests; in the sheep blowfly *Lucilia cuprina,* Li and colleagues developed a transgenic strain with a reporter fluorescent marker overexpressed in female larvae, enabling sex-sorting of early larvae [31]. The tetracycline-repressible marker expression was female-specific due to the splicing of a *tra* intron, highlighting the fact that many sex-determining genes may prove useful in the development of new sexing methods.

Conditional Female Death Early in Development

Conditional female lethality is commonly used in order to generate sexing strains. Several of these methods are based on female-specific expression of a tetracycline transactivator (tTA) driving the expression of a proapoptotic transgene [32–34]. In these three studies, insects showed normal hatching rates and sex ratios when reared in media containing tetracycline, but a 50% hatching rate and male-only generations were obtained when tetracycline was absent. In *Anastrepha suspensa,* the Caribbean fruit fly, Schetelig and colleagues hatched 30 000 transgenic eggs and obtained 100% males, suggesting that a mass-rearing application would be possible [33]. Recently, the *C. capitata* transgenic strain, FSEL#32, was compared to the current reference VIENNA 8 [35] in a mass rearing context. FSEL#32 exhibited higher productivity and similar mating competitiveness, despite having lower pupal weights [36].

RNAi techniques have been tested to cause female lethality by silencing female-specific exons of sex-determination genes. In *Bactrocera dorsalis* embryos, RNAi against transformer and transformer-2 (*tra, tra-2*) resulted in 96% of males and 4% of sterile, nonmating, intersex individuals, though the hatching rate dropped to 15.6% [37]. A similar silencing of *tra-2* in *Ae. aegypti* larvae, either by soaking them in a dsRNA mixture or by feeding bacteria expressing dsRNA, led to a survival rate of about 50% and a sex bias towards males of 97.6% [38]. The female isoform of *doublesex* (*dsx*) has also been targeted in *Ae. aegypti.* Survivors were 97% males, the remaining females being sterile and mostly unwilling to blood-feed [39].

While most conditional female lethality approaches have focused on disrupting female-associated genes, Krzywinska *et al.* [40] experimented with an alternative approach: by inducing ectopic expression of Yob, a gene that may facilitate male-specific splicing of *dsx* in *An. gambiae*

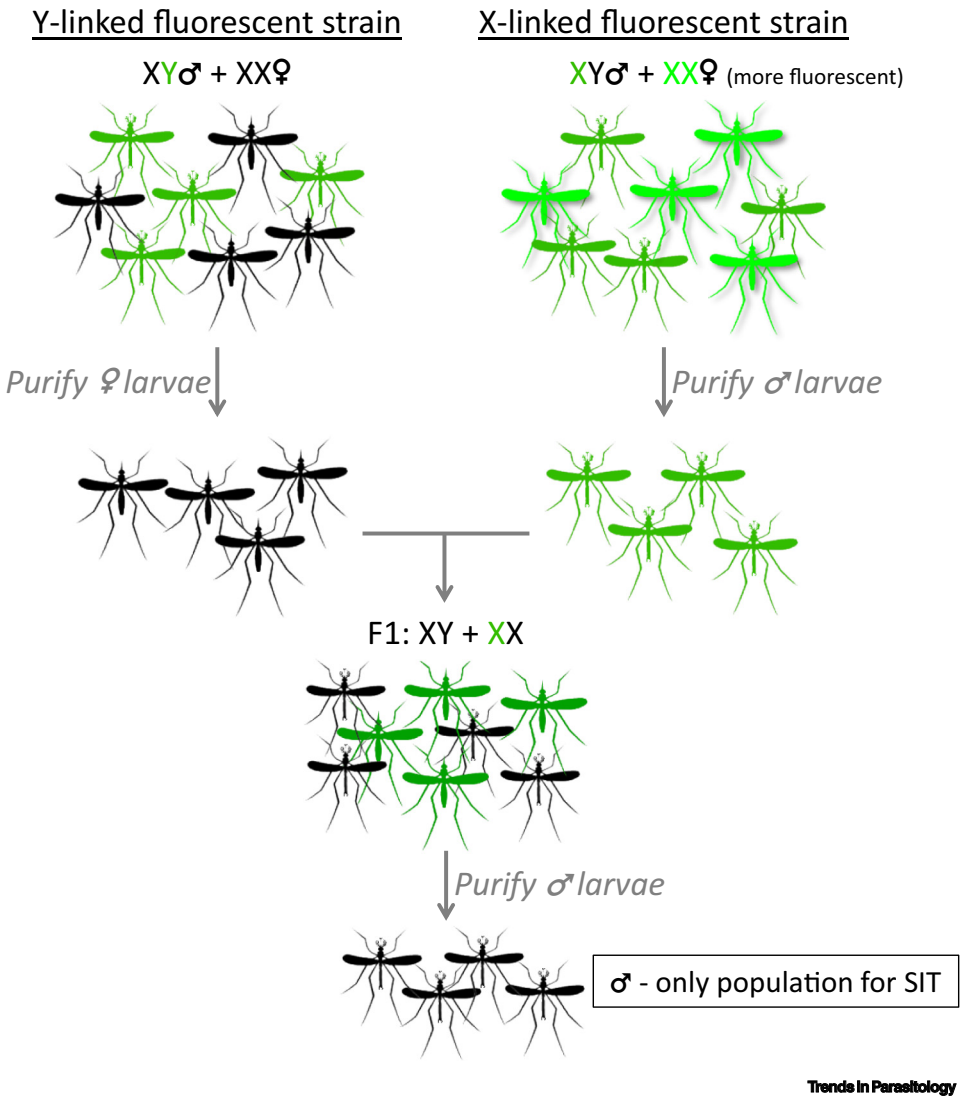

Figure 1. Principle of Using Two Transgenic Sexing Strains to Generate a Population of Nontransgenic Males. Two transgenic strains are employed: one expresses a fluorescent marker from the Y chromosome (only males are transgenic), the other from the X chromosome (XX females are more fluorescent than XY males). Fluorescence-based automated sorting allows the production of a population of wild-type females from the first line and of hemizygous fluorescent males from the second. These are crossed together. In their F1 progeny, males are nonfluorescent as they inherited a wild-type Y chromosome from their fathers and a wild-type X chromosome from their mothers, whereas their sisters are fluorescent. A new round of automated selection allows the purification of a male-only, nontransgenic population.

and *An. arabiensis*, they observed that all Yob mRNA-injected individuals that survived were indeed male, as females injected with Yob mRNA died during development.

Generating Males and Intersex Individuals

A less cost-effective approach to early and complete sex-sorting would be to generate a population comprised of males and partially masculinized females. Females produced by these methods are usually sterile and, in mosquitoes, nonbiting. These females would still compete with males for food and space in the rearing facility, but the released population would be more efficient in the field.

Following their previous promising results with Yob-mediated female killing [40], Krzywinska and Krzywinski produced transgenic *An. gambiae* that overexpressed *Yob* in the germline [41]. This strain produced a male-biased population with 75% males and 25% masculinized females of decreased viability, complete infertility, and various levels of intersexual morphological defects, indicating that transgenic construct optimization may soon lead to the full conversion of genetic females into functional males. More recently, Li and Handler injected CRISPR-Cas9 components to knock down expression of *transformer-2* in *A. suspensa* eggs, producing 42% males, 47% intersex individuals, and 11% of females, of which only 13% survived to adulthood [42]. This opens the possibility to develop a transgenic conditional sexing system in this species.

Remarkably, conversion of genetic females into phenotypic males was recently achieved in *Ae. aegypti* [43] using the male factor *Nix*. A final step to convert these encouraging results into sexing strains producing only pure males would be to develop a system for conditional expression of the male factors.

Late-Acting Sex-Separation Methods

Removal of females at late larval or pupal stage requires more space and maintenance in a factory setting, which can increase the cost of area-wide control programs. However, such methods are used in many SIT programs, and are still being developed (Table 2).

Automated Sex-Separation in Late Developmental Stages

Automated sex separation of pupae has been achieved in several plant pests such as *B. dorsalis, Bactrocera carambolae, Zeugodacus cucurbitae* and *A. ludens* using Genetic Sexing Strains (GSS) with pupal color mutation [44–47]. Sorting by this method was nearly 100% efficient, and these strains showed good competitiveness [45,48,49] in field trials. Because the mutations

Table 2. Late-Acting Methods for Sexing in Diptera since 2003

Outcome	Technique[a]	Sorting mechanism	Species	Refs
Visual separation	Chromosomal translocation to the Y chromosome	Pupal color dimorphism	*Zeugodacus cucurbitae, Bactrocera dorsalis, B. carambolae, Anastrepha ludens*	[44–46]
	Transposase-mediated plasmid integration (*piggyBac*)	Fluorescent markers integration near *β-2-tubulin* gene	*Anopheles stephensi, Aedes aegypti, Anopheles arabiensis*	[26,50,51]
		Fluorescent markers on Y chromosome	*Ceratitis capitata*	[52]
	Near-infrared photography	Pupal dimorphism	*Glossina palpalis gambiensis, G. pallidipes*	[53,54]
	Computer vision analysis	Pupal dimorphism	*Aedes albopictus, Ae. aegypti, Ae. polynesiensis*	[55]
	Protandry selection	Collecting first pupations	*Ae. albopictus*	[56]
Female lethality	Transposase-mediated plasmid integration (*piggyBac*)	Tetracycline-repressible system: Sex-specific splicing of *transformer* + lethality effector	*C. capitata, B. oleae, Lucilia cuprina, Cochliomyia hominivorax*	[13,57–59]
		Tetracycline-repressible system: Female-specific *actin-4* regulatory region	*Ae. aegypti, Ae. albopictus, An. stephensi*	[60–62]
	Chromosomal translocation to Y chromosome	Dieldrin resistance	*An. arabiensis*	[64,69,70]
	Chromosomal translocation to male-locus	Dieldrin resistance	*Ae. albopictus*	[71]
	Infected blood meals	Ivermectin insecticide in blood meals	*An. arabiensis*	[72]
	Cas-9 and CRISPR guides in different lines	Simultaneous knockdown of *β-tubulin* and *sex lethal*	*Drosophila melanogaster*	[73]

[a]Techniques are detailed in Figure S1 in the supplemental information online.

were associated with chromosomal translocations (Figure S1), these strains are semisterile. Where color mutations are difficult to find or manipulate, late-stage sexing can be achieved using fluorescent marker proteins under the control of sex-specific promoters. Markers linked to the *β-2-tubulin* gene promoter allowed good sex separation in late larvae or pupae in different mosquito vectors, such as *An. stephensi, Ae. aegypti* and *An. arabiensis* [26,50,51], even though automation by COPAS was low in throughput due to the relatively large size of half-grown larvae and their strong autofluorescence. In *C. capitata*, Alphey and colleagues produced two transgenic sexing lines with strong fluorescence at the late larval stage with correct sorting in 97.5% to 100% of pupae screened (n = 396 and n = 235 pupae, respectively) [52]. Developing automated sorting methods for each of these strains is an important prerequisite for sex separation at operational scale.

Nontransgenic methods, based on imaging technologies, are also in development and could prove valuable in locations where genetic modification is rejected. In the tsetse flies *Glossina pallidipes* and *G. p. gambiensis*, near infrared (NIR) imaging allowed sex separation of wild-type (WT) pupae based on sexual dimorphism with 80–100% accuracy [53,54]. Similarly, an automated pupal size estimator measuring lateral profile areas from Grupo Tragsa (Spain) has been tested on different *Anopheles* and *Aedes* species and strains [55]. Ensuring <1% female contamination, *An. arabiensis* could not be sorted efficiently, but *Aedes* sorting resulted in 65 and 98% of male recovery, surpassing the performance of sorting plates.

Bellini and colleagues tested a very different approach in *Ae. albopictus*: over several generations, they selected males and females to accentuate differences in pupation time. After ten generations, they produced a strain in which the 28% earliest pupae were 99% male [56], which is similar to sieving plates' efficiency. This method illustrates that other phenotypes could serve as future targets for genetic-based sexing approaches.

Conditional Female Death in Late Developmental Stages

Female lethality in late larval to adult stages has also been considered in several species. A widely used conditional method involves the tTA, either controlling the expression of a lethal transgene or itself triggering lethality. Fu and colleagues successfully developed such a *C. capitata* strain yielding full female lethality by using sex-specific *tra* intron splicing to control the expression of the lethality construct [57]. The same system allowed establishing a *Bactrocera oleae* RIDL strain carrying sex-specific fluorescence, genetic sterility, and conditional female-lethality [58]. In cage tests, this strain efficiently eliminated a WT population using weekly releases of transgenic males. Female-specific splicing of *transformer* was also used in *L. cuprina* [59] and in *C. hominivorax* [13] in combination with proapoptotic genes. One of the *C. hominivorax* strains is currently under evaluation for a mass-rearing program [12].

In several mosquito species, the female-specific expression of *actin-4* was exploited to conditionally express lethal effectors. As *actin-4* is expressed in female indirect flight muscles, the obtained phenotype is flightless females, not death. Such a system was developed in *Ae. aegypti*, *Ae. albopictus*, and *An. stephensi* with full penetrance of the flightless phenotype in the absence of tetracycline [60–62]. Although this system seems effective in laboratory studies, it has been suggested that tetracycline affects gut microbiota and impairs *An. stephensi* fitness, and may render inadvertently released females more susceptible to *Plasmodium falciparum* infection [63].

In 2012, Yamada and colleagues developed the ANO IPCL1 strain, an *An. arabiensis* strain in which the dieldrin-resistance allele was translocated to the Y chromosome so that males are resistant to dieldrin and females susceptible [64]. This strain presents low productivity, male

recovery being 13% of the initial number of eggs [65–67] with a risk that released males spread highly toxic dieldrin into the environment [68]. However, this strain has been backcrossed into different *An. arabiensis* genetic backgrounds [69,70]. In 2018, Lebon and colleagues developed a similar strain in *Ae. albopictus,* TiCoq, with a sex-sorting efficiency of 98% [71].

Given that only female mosquitoes blood-feed, toxicant-infused blood meals have also proven effective for sexing [72]. With ivermectin provided at 7.5 p.p.m. in blood meals, all *An. arabiensis* females died after 4 days without compromising the males. Ivermectin is, however, ejected in female feces, which causes the contamination of all rearing equipment, a major disadvantage if this system is used in mass-rearing conditions.

Recently, Kandul and colleagues described a system producing 100% sterile males in *D. melanogaster*, females dying mainly at the late larval stage [73]. The strategy involves crossing a strain expressing Cas9 enzyme with another expressing β-tubulin (*β-tub*) and sex lethal (*sxl*) CRISPR targets. This study sets a proof-of-principle that it is possible to get both sterilization and sex-sorting in the F1 generation, keeping mating efficiency similar to that of the wild type. However, this approach would require another perfect sexing method in the starting generation to sort males and females to establish the correct cross. Moreover, the researchers propose to release eggs so that the female larvae compete for food in density-dependent species but it is hardly possible in *Aedes* mosquitoes that have multiple small breeding sites, and such strains cannot be used for pests whose larvae damage crops [74].

Elements for Future Successful Sex Sorting

Acceptability of Sex-Sorting Technologies by the Public and by Governments

While SIT has been used in pest control for 60 years, it is not widely understood by the public, especially with respect to its ability to control vector populations. On La Reunion island, where a trial is planned against *Ae. albopictus*, a poll showed that only 34% of the island inhabitants knew about the technique[i]. However, when informed about the SIT principle, 61% supported the release of wild-type, irradiated sterile males. Releases of **genetically modified** (**GM**) mosquitoes has historically faced more opposition than using sexually dimorphic traits: some had to be cancelled due to important public concerns, all related to lack of information and communication [75]. Antonelli and colleagues found that, depending on the terminology used to present transgenic mosquitoes, public opinion varies [76]. Consequently, even though their genetic status is of less relevance when releasing sterile insects (see 'organism' in the glossary), the release of strains obtained by classical mutagenesis might appear less worrisome than transgenic strains. Concerns have also been raised by the scientific community itself when Oxitec released transgenic mosquitoes for a field trial in the Caribbean [21,75,76]. Similarly, several governments are opposed to the release of genetically modified insects on their territory. Scientists must take these concerns into account when developing a new technology.

A Broad Range of Promises for Sexing Vector Diptera

For screwworm eradication programs, Concha and colleagues calculated that production expenses represent 20% of total program costs [13]. In Figure 2, we compare the proposed production methods to the current standard for seven key parameters that affect costs and feasibility: sorting stage, male recovery rate, female contamination rate, sorting speed, initial investment, treatment cost, and strain characteristics. Sorting stage, male recovery rate, and female contamination rate together influence the number of insects to be reared, and therefore the cost and space required to achieve the desired efficiency. Male quality (survival and competitiveness) could also affect efficiency. Here, we assume that it is equivalent in all methods. Sorting speed, whether manual or automated, can be decisive for feasibility in a mass-rearing context.

Figure 2. Efficiency and Cost of the Reviewed Sexing Methods Compared to the Currently Used Ones in Vectors. For each species, the method indicated in bold and its parameter values is the current standard. Other methods developed for the same species are compared, point to point to the standard, with green rectangles when performance is improved, or red rectangles when it is decreased. No rectangle means that the value is comparable to the reference. Values are indicated above the rectangle or on the middle line when they differ from the reference. All values are from the literature. The asterisk (*) means that both males and females are released and that the flightless females survive a few days on the release site. Time, in hours, is calculated for one device, or for one operator sex-sorting 1 million males. Investment is given in orders of magnitude: '+' being 10^3–10^4, '++' being 10^5 and '+++' being 10^6. Treatment cost is given as presence ('+') or absence ('0'). Abbreviations: L1, 1st instar larvae; L4, 4th instar larvae; WT, wild type; TR, transgenic; CM, obtained by classical mutagenesis; CT, chemically treated; NC, noncommunicated; *Glossina*, *Glossina p. gambiensis* and *Glossina pallidipes*. See also [15,19,26,28–30,50,51,53–55,59,60,62,64,69,70,72].

For instance, *Anopheles* mosquitoes are currently sorted manually at a speed of 500 insects/hour/trained worker. Sorting one million males would therefore take about 4000 hours for a single operator. We also considered the initial investment of sorting device and the cost of sorting supplies, if any. For example, imaging and sorting devices represent a significant initial investment.

Methods causing female lethality avoid initial costs but may involve daily costs of chemical treatment (dieldrin, tetracycline) as well as the treatment of contaminated rearing water. Finally, since strain characteristics can influence their social and legislation acceptability, we included this parameter by considering that WT strains would be preferable to conventional GSS and transgenic strains.

In mosquitoes, since females cause nuisance and transmit pathogens, scalable sex-sorting methods must achieve greater than 99% female removal. Accordingly, for mosquitoes, Figure 2 covers strategies with a female contamination rate <1%. Figure 2 shows that there is no perfect solution that could decrease all costs and avoid a large investment while being readily acceptable by the public and legislators. Most early sex-sorting methods, and many late-acting ones, rely on transgenic technologies. These are promising in terms of rearing cost-efficiency, but their upscaling might be restricted by negative public perception or regulatory prohibitions. Pupal sorters for *Aedes* mosquitoes [55] and NIR imaging for tsetse flies [54] also appear as promising approaches for these species, though their speed and cost are currently prohibitive. In *Anopheles* mosquitoes, not amenable to such approaches, the crossing scheme to obtain WT males as presented in Figure 1 might help to overcome this obstacle. Classical GSS similar to pupal color traits in flies [44–46] might be useful for automated sorting based on known mutant alleles such as *stripe* [77] or *redeye* [78], when transgenic approaches must be avoided. Recently, Ndo and colleagues isolated a temperature-lethal mutation [79] that could also be used for building a GSS in *Anopheles*. In situations where transgenesis is not a problem, the use of tTA driving expression of a proapoptotic transgene was demonstrated to cause female death in early larval stages in several plant and livestock pests [32–34] and could be investigated for sexing vector species.

Here, late sex-sorting was penalized, while for approaches such as RIDL, it is a desired characteristic so that released female larvae compete for food and space with wild larvae before dying. Moreover, RIDL systems would be more efficient than conventional SIT for the same number of released insects if the same competitiveness could be achieved [80]. Similarly, sex-ratio distortion strains carrying X chromosome-shredding systems [24,25] were not extensively discussed in this review since they result in nonconditionally male-biased populations. However, such systems have proven to be very efficient for genetic control in large cage trials [81]. Provided the strains can be maintained in a mass-rearing facility, repeated releases could be 16–3000 times more efficient than SIT and 2–70 times more than RIDL [82].

Concluding Remarks

Sexing Diptera has received a lot of attention over the past 15 years. Proposed methods include early and late sexing with variable outcomes in terms of sorting efficiency. Other parameters, including sorting technology and treatment cost, as well as strain characteristics, influence their feasibility and acceptability by the public and governments. To enrich the toolbox for vectors, recent developments in pest sexing are a valuable source of inspiration. Further research is needed on theoretical aspects of sex determination and practical development of sexing strains to foster progress in genetic vector-control programs, Novel methods will need to meet sorting efficiency but also social and regulatory acceptance criteria (see Outstanding Questions).

Outstanding Questions

What are the other factors involved in sex determination in Diptera, and how can we use them for building new sexing strains? For species with highly repetitive genomes, can long-read assemblies help us to find new targets?

Are next-generation pupal sorters for *Aedes* mosquitoes and tsetse flies scalable alternatives to the current sexing methods?

High-throughput sexing of 1st stage larvae by COPAS fluorescence recognition was successfully achieved in *An. gambiae*. Can it be applied to other *Anopheles* mosquitoes with the same efficiency? How to transfer this technology to other Culicidae that are quite different genetically?

How are public opinion and country laws going to turn in coming years regarding transgenic insect releases? Should such approaches be continued or abandoned?

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Resources

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